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13. ABSTRACT (Maximum 200 Words)

Breast cancer is the second deadliest cancer in US women, with estimated 182,800 new cases yearly. PTEN has been characterized as a tumor suppressor gene and found deleted or mutated in many human tumors, including breast, and functions to negatively regulate cell growth, migration, etc, through down-regulation of downstream mediators, such as Akt, etc. Germline PTEN mutations are associated with Cowden syndrome, characterized by increased risk of developing breast cancer. PTEN expression has a positive ER and PR status in primary breast cancers. Whereas, approximately 65% of tumors tested are positive for ER and PR and 75%-80% positive for AR. Androgen, through AR, inhibits mammary carcinoma growth in animal models and is used clinically to influence breast cancer progression. AR germline mutations can cause partial androgen insensitivity. Combined with BRCA1 germline mutations associated with earlier age onset breast cancer, AR functional changes are implicated in breast cancer, suggesting PTEN and AR play roles in breast cancer progression. However, detailed correlations remain unknown. We propose the PTEN pathway, by AR interaction, results in AR-mediated cell growth modulation, and provides a new molecular mechanism of PTEN-mediated AR suppression signaling pathways. Our studies may provide new gene therapies/drug designs for breast cancer patients.

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Introduction

(See also abstract page and Introduction to first year's report.)

It has been reported that PTEN is one of the most commonly mutated genes in human cancers, including breast cancer, and that reintroduction of PTEN into breast cancer cells results in growth suppression. Also, patients with loss or reduction of AR function caused by mutations are predisposed to the development of breast cancer. This suggests that both PTEN and AR play important roles in the progression of breast cancer. However, the correlation between PTEN and AR in breast cancer remains unknown. Therefore, this study provides not only a new molecular mechanism of PTEN-mediated AR suppression signaling pathways, which implicates the suppression of cell growth in breast cancer, but also has therapeutic implications, such as gene therapy or drug design, for breast cancer patients in the future. Our objective is identify a new PTEN pathway by direct interaction with AR that may result in the suppression of AR-mediated cell growth. The detailed mechanism of how PTEN can repress AR transactivation will also be dissected. The scope of our work includes the Aims 1) to determine that PTEN suppresses AR transactivation and causes growth suppression in MCF-7 cells., 2) to demonstrate that the direct interaction of PTEN and AR represents a new signaling pathway and to determine whether PTEN directly dephosphorylates AR as a PTEN phosphatase substrate, and 3) to determine the molecular mechanisms of how PTEN can repress the AR transactivation. These aims will be accomplished by a) determining the suppressive effect of PTEN and PTEN-C124S on AR transactivation and the growth of MCF-7 cells, b) determining the interaction domains between PTEN and AR, c) proving the *in vivo* interaction of PTEN and AR in co-immunoprecipitation assay in MCF-7 cells, d) performing the co-localization of PTEN and AR in MCF-7 cells to understand in which compartment PTEN functions on AR, d) establishing the experimental conditions of in-blot phosphatase assay to investigate whether PTEN has phosphatase activity on AR, and e) determining that PTEN reduces AR protein expression.

BODY Statement of Work

Task 1(1 year): Determine the suppressive effect of PTEN and PTEN-C124S on AR transactivation and the growth of MCF-7 cells.

a. Determine endogenous AR-targeted p21 mRNA expression in MCF-7 cells transfected with PTEN and PTEN-C124S
b. Construct plasmids such as pTet-on-PTEN and pTet-on-PTEN-C124S
c. Establish MCF-7 cells stably transfected with vector, PTEN, and PTEN-C124S by neomycin selection
d. Determine MTT growth assay and Northern Blotting detecting p21 mRNA expression

Task 2 (1 year): Determine the interaction domains between PTEN and AR

a. Construct series of PTEN cDNA fragments into GST expression vectors
b. Construct series of AR cDNA fragments into pET-28c expression vectors
c. Determine the AR-binding sites of PTEN and PTEN-binding sites of AR in GST pull-down assay

Task 3 (0.5 year): Prove the *in vivo* interaction of PTEN and AR in co-immunoprecipitation assay in MCF-7 cells.

Task 4 (0.5 year): Perform the co-localization of PTEN and AR in MCF-7 cells to understand in which compartment PTEN functions on AR.

Task 5 (0.5 year): Establish the experimental conditions of in-blot phosphatase assay to investigate whether PTEN has phosphatase activity on AR.

Task 6 (0.5 year): Establish the experimental conditions of in-blot phosphatase assay to investigate whether PTEN has phosphatase activity on AR.

a. Determine whether PTEN influences AR protein expression by enhancing AR degradation in pulse-chase assay.
b. Determine whether the AR instability is enhanced by dephosphorylation of AR by PTEN or by caspase- or ubiquitinase-mediated protein degradation.

c. Determine whether PTEN also influences AR protein expression at transcriptional or translational levels in Nuclear Run-on assay.

Progress towards Task 1, Task 2 and Task 3: Previous results (See previous annual report) showed that PTEN can suppress AR transcriptional activity, and PTEN endogenously interacts with AR in MCF-7 breast cancer cell lines. Because MCF7 cells contain both PTEN and AR, we can not directly use this cell line to demonstrate the changes of AR nuclear translocation in the absence or presence of PTEN. Therefore, we studied a common used cell line COS-1 which does not contain PTEN and AR. (**see Fig. 1**). We can look at how PTEN influences AR simply by transient transfection of AR with or without PTEN. We found that PTEN could reduce AR protein levels in COS-1 cells.

Also, we could apply AR-D, an interaction inhibitor, to see if it could block the PTEN effect on AR. Once we find the positive results using COS-1 cells, we will study them in CF7 cells using PTEN siRNA to suppress endogenous PTEN to see if it affects AR translocation. As shown in the figures, we indeed saw this suppression. Next to test the suppression efficiency of several PTEN siRNA vectors that we have constructed on endogenous PTEN expression, we applied them in 293T cells because this cell line contains endogenous PTEN and most importantly, the transient transfection efficiency is very high (more than 50%) using SuperFect (Qiagen). As shown in the Fig, the PTEN siRNA (the most efficient one) can efficiently suppress PTEN expression and increase the AR amount and transactivation, indicating that endogenous PTEN normally inhibits AR expression and transcriptional activity. We have demonstrated that overexpression of PTEN promotes AR degradation and suppresses AR activity. To avoid the obtained observations resulting from overexpression, we used small interfering RNA (siRNA) to block endogenous PTEN and examined whether the AR protein levels and transcriptional activity would be affected by downregulating). As shown in **Fig. 2A**, transient transfection of PTEN siRNA reduced endogenous PTEN protein levels up to 50-60%, which correlated with the transfection efficiency (around 50%) in 293T cells. As expected, reduction of PTEN expression enhanced AR protein expression in the presence and absence of androgen (Fig. 2A). Likewise, PTEN siRNA dose-dependently enhanced AR transactional activity (Fig. 2B). These results suggest that endogenous PTEN is a negative regulator for controlling AR protein stability and transcriptional activity.

Next, we will apply this PTEN siRNA in MCF7 cells to see if suppression of PTEN expression by PTEN siRNA can affect AR as well as ER and cell growth in breast cancer cells. Presently and into the next year we are looking for a way to transfect PTEN siRNA into MCF7 cells at maximum levels. We are testing conditions of electroporation. If we fail, we will try using flow cytometry to sort out transfected cells.

Progress towards Task 4. To further investigate whether PTEN can co-localize with AR and then influence AR nuclear translocation we assessed the immunocytofluorescence staining in COS-1 cells. Again, we can not directly use this MCF-7 to demonstrate the changes of AR nuclear translocation in the absence or presence of PTEN. See Fig. 3 discussion page. We will be performing these studies in MCF-7 cells in the near future.

Progress towards Task 5: None

Progress towards Task 6. 6a only. To determine whether PTEN influences AR protein expression by enhancing AR degradation in pulse-chase assay in COS-1 cells. Again, we can not directly use this MCF-7 to demonstrate the changes of AR nuclear translocation in the absence or presence of PTEN. As shown in Fig. 2B, PTEN clearly reduced the half-life of newly synthesized [35S]-AR 4 to 5-fold and accelerated AR degradation.

Note: Due to the continuing problems studying the MCF-7 cell line as regards the PTEN, we may need to consider a change in our scope of work and begin study of other breast cancer cell lines.

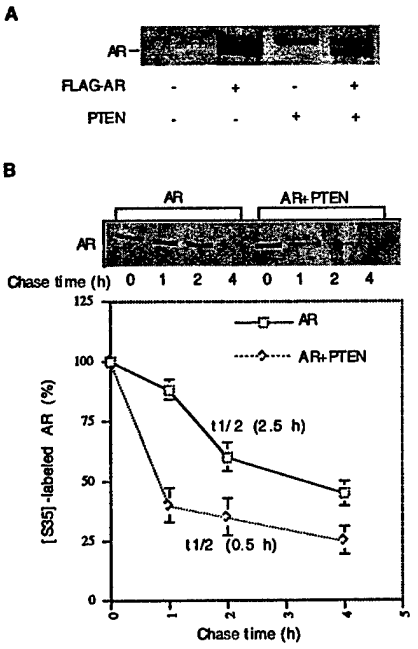


Fig. 1 A. COS-1 cells were transfected with AR with a flag epitope in front of the AR sequence, along with pCDNA3 or PTEN in 10% CDS media for 16 h. The cells were harvested for Western blot analysis. B. COS-1 cells were transfected with AR along with pCDNA3 or PTEN in 10% CDS media for 16 h. The cells were then pulsed with [35S]-methionine for 45 min in the presence of 10 nM DHT and harvested at different chase times as indicated. The cell extracts were immunoprecipitated with AR antibody and subjected to SDS-PAGE followed by autoradiography.

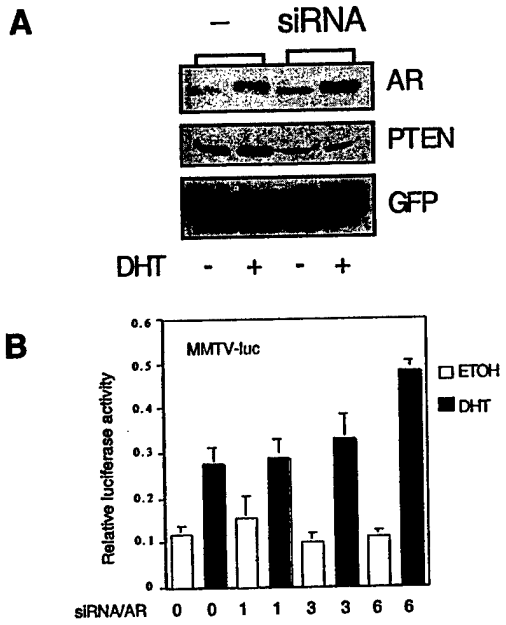
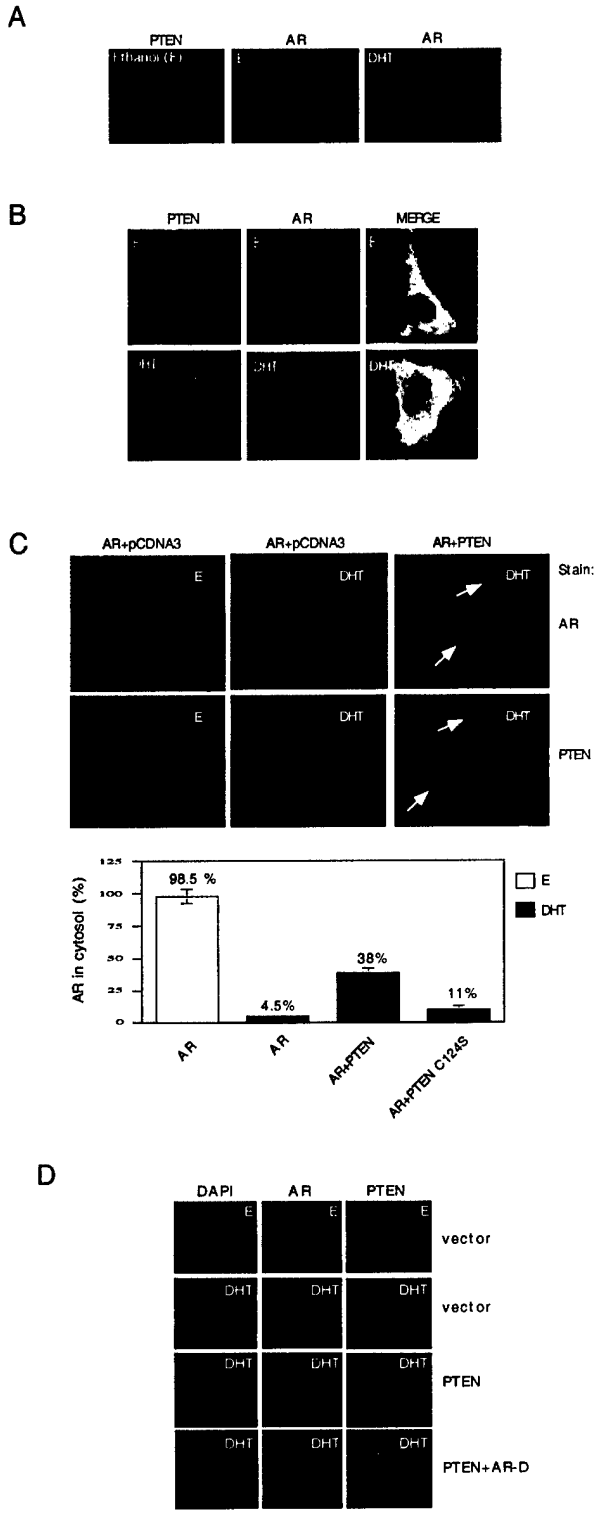


Fig. 2. Endogenous PTEN negatively regulates AR protein stability and transcriptional activity. (A) 293T cells were transfected with PTEN siRNA along with AR for 24 hours, followed by ethanol or 10 nM DHT treatment for another 24 hours, and harvested for Western blot analyses. (B) 293T cells were transfected with various amounts of PTEN siRNA along with AR and MMTV-luc for 24 hours, followed by ethanol or 10 nM DHT treatment for another 24 hours, and harvested for luciferase assays.

PTEN co-localizes with AR *in vivo* and prevents AR nuclear translocation.



(A,B) The COS-1 cells were transfected with AR or PTEN in 10% CDS media for 16 h and treated with ethanol or 10 nM DHT for another 16 h. The cells were fixed and stained with AR and PTEN antibodies, followed by examination with confocal microscopy. The green and red colors represent PTEN and AR staining, respectively, and the yellow color represents PTEN and AR colocalization. (C) The arrows indicate PTEN positive cells, which show AR located in the cytosol. At least 150 cells were scored for each sample, and data are means ± s.d. from three independent experiments. (D) The COS-1 cells were transfected with AR in combination with plasmids, as indicated for 16 h, followed by 10 nM DHT treatment for another 16 h. The cells were fixed for immunostaining.

The interaction between PTEN and AR was also analyzed by the subcellular co-localization study, using fluorescence immunostaining. As shown in Fig. 3A, the fluorescent FITC-stained PTEN was mainly located in the cytosol, but small amounts of PTEN were also found in the nucleus. Similar to the FITC-stained PTEN, Texas-RED-stained AR was also mainly located in the cytosol in the absence of androgen, but androgen treatment caused AR nuclear translocation (Fig. 3A). Fig. 4B further demonstrates that PTEN could co-localize with AR in the presence or absence of androgen. Interestingly, we found that PTEN significantly blocked AR nuclear translocation in response to androgen and increased the AR retention (from 4% to 38%) in the cytosol. In contrast, PTEN C124S only showed a slight inhibition of AR nuclear translocation (Fig. 3C).To further prove that PTEN suppression of AR function may go through direct PTEN-AR interaction, we utilized AR-D, which can interact with PTEN and disrupted the interaction between AR and PTEN in the CWR22R cells (Fig. 3D), for functional studies. Our results further showed that AR-D could dramatically reduce PTEN-mediated inhibition of AR nuclear translocation (Fig. 3E)

Key Research Accomplishments:

- We found that PTEN could reduce AR protein levels in COS-1 cells.
- PTEN siRNA (the most efficient one) can efficiently suppress PTEN expression and increase the AR amount and transactivation, indicating that endogenous PTEN normally inhibits AR expression and transcriptional activity
- We have demonstrated that overexpression of PTEN promotes AR degradation and suppresses AR activity.
- Transient transfection of PTEN siRNA reduced endogenous PTEN protein levels up to 50-60%, which correlated with the transfection efficiency (around 50%), reduction of PTEN expression enhanced AR protein expression in the presence and absence of androgen, and PTEN siRNA dose-dependently enhanced AR transactional activity, suggesting that endogenous PTEN is a negative regulator for controlling AR protein stability and transcriptional activity.
- PTEN can co-localize with AR and then influence AR nuclear translocation we assessed the immunocytofluorescence staining in COS-1 cells.
- In pulse-chase assays, PTEN clearly reduced the half-life of newly synthesized [35S]-AR 4 to 5-fold and accelerated AR degradation.

Reportable Outcomes: None.

Conclusions: Our research to date has determined much about the PTEN effects on the AR, including reducing AR protein levels, inhibiting AR expression and transcriptional activity, the overexpression of PTEN promotes AR degradation and suppresses AR activity, endogenous PTEN is a negative regulator for controlling AR protein stability and transcriptional activity, and that PTEN can co-localize with AR and then influence AR nuclear translocation in several cell lines including some study in MCF-7 breast cancer cell lines. We recommend the slight change in scope of our work by the addition of breast cancer cell lines other than the MCF-7. If we can adapt these studies better to the MCF-7 cells or other breast cancer cell lines we may uncover molecular mechanisms of the suppression of AR by PTEN, which in turn may imply tumor growth suppression and lead to better therapeutic strategies, for instance gene therapy, for breast cancer patients in the future.

References: None

Appendices: None